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Differential Regulation of a Hydroxyproline-Rich Glycoprotein Gene Family in Wounded and Infected Plants

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We have characterized three different transcripts induced by fungal elicitor, wounding, or infection which encode apoproteins of cell wall hydroxyproline-rich glycoproteins involved in plant defense against infection. The proteins encoded by two of these transcripts contain a proline-rich domain involving tandem repetition of the 16-amino-acid unit Tyr_3-Lys-Ser-Pro_4-Ser-Pro-Ser-Pro_4. The third transcript encodes a protein with a proline-rich domain involving a variant of this 16-mer canonical repeat: Tyr_3-His-Ser-Pro_4-Lys-His-Ser-Pro_4. Each transcript is encoded by a separate gene present at single or low copy number in the haploid genome. These transcripts exhibit markedly different patterns of accumulation in different stress conditions, indicating the operation of several distinct intercellular stress signal systems in higher plants.

The natural resistance of plants to disease involves an array of inducible defense responses, including the synthesis of phytoalexin antibiotics and the cell wall structural polymer lignin, accumulation of cell wall hydroxyproline-rich glycoproteins (HRGP), and increases in the activities of hydrolytic enzymes such as chitinase (4, 38). These responses can also be induced by treatment of suspension-cultured cells with glycan and glycoprotein elicitors isolated from microbial cell walls and culture fluids (14, 17). Genes encoding proteins involved in these resistance mechanisms have been isolated, and cloned sequences have been used in RNA blot hybridization and nuclear runoff transcription experiments to show that elicitors cause marked transcriptional activation of defense genes in suspension-cultured bean (*Phaseolus vulgaris*) and parsley (*Petroselinum hortense*) cells (9, 28). Defense gene activation, which in some cases occurs within 2 to 3 min of elicitor treatment, is part of a massive change in the pattern of mRNA and protein synthesis underlying the induction of defense responses (13).

Although elicitor-treated cell cultures provide a simple and convenient experimental system, they do not display the genetically controlled race-cultivar specificity which underlies a large number of plant-pathogen interactions, including many agriculturally important diseases (15). Moreover, studies with cell cultures cannot provide information on the spatial orchestration of defenses in relation to localized damage or pathogen ingress. Therefore, we have extended the analysis from elicitor-treated cells to examine the activation of defense genes in bean hypocotyls in response to mechanical wounding and during race-cultivar-specific interactions with the fungus *Colletotrichum lindemuthianum*, the causal agent of anthracnose (5, 6, 28).

In a genetically incompatible interaction after fungal penetration of the first epidermal cell, there is a marked early induction of defense gene transcripts in tissue directly underlying the site of spore inoculation, correlated with expression of localized hypersensitive resistance and complete inhibition of further fungal growth (6, 28). Moreover, transcription of defense genes is also stimulated at a distance in uninfected tissue, associated with the establishment of induced immunity and protection from subsequent attack by normally virulent pathogens. In contrast, in a genetically compatible interaction with a virulent race of *C. lindemuthianum*, there is no marked early response to fungal penetration; instead, widespread accumulation of defense gene transcripts is observed in the later stages of the interaction after extensive fungal growth has occurred. This delayed activation of defense genes is correlated with the initial appearance of anthracnose lesions and represents a lesion limitation response. Under physiological conditions favorable to the host this response can prevent complete rotting of the hypocotyl and death of the plant even though the interaction is genetically compatible (2). Wounding of hypocotyl tissue under sterile conditions also causes a rapid activation of defense genes which, as with infection, leads to accumulation of transcripts in distant undamaged tissue as well as in tissue immediately adjacent to the localized perturbation (6, 28).

These studies focus attention on the organization and structure of defense genes and the molecular mechanisms underlying transcriptional activation in response to wounding and infection. Since plant defenses are elaborated in a number of very different circumstances, a key question is whether plants utilize an array of cues for defense gene activation such that different signal transduction systems operate in the early stages of the incompatible interaction compared with the later stages of the compatible interaction or wounded tissue. A number of defense genes are organized in small multigene families (8, 36); hence the operation of a set of distinct signal transduction systems might be reflected in selective activation of individual members of such gene families in different stress conditions. In the present paper we have characterized a number of cDNA clones containing sequences encoded by a set of elicitor-induced HRGP genes. We have used these clones as hybridization probes to compare the patterns of accumulation of the different HRGP transcripts in response to wounding and infection and hence to delineate the complexity of stress signal systems in wounded and infected plants.

HRGPs are major structural components of plant cell walls (26, 27). In addition to hydroxyproline (Hyp), cell wall HRGPs are rich in serine (Ser), valine (Val), tyrosine (Tyr), and lysine (Lys) and contain a characteristic pentapeptide repeating sequence, Ser-Hyp_4 (see reference 11 for a review). Recently, a carrot genomic clone encoding a cell wall HRGP was isolated, sequenced, and shown to contain 25

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Ser-Pro_4 repeat units, the unhydroxylated precursors of the Ser-Hyp_4 repeat units, distributed throughout the 306-amino-acid coding region (10). The carbohydrate moiety of cell wall HRGPs is composed largely of short oligo-arabinosides attached O glycosidically to most of the hydroxyproline residues and to a much lesser extent of galactose, which is O glycosidically linked to some of the serine residues. The mature HRGPs are hypothesized to form extended helical rods and become insolubilized in the cell wall, perhaps through the formation of isodityrosine bonds (11). HRGPs may function in defense as specific microbial agglutinins (29, 32) or as structural barriers, either directly or by providing sites for lignin deposition (22).

In previous studies, tomato HRGP genomic sequences were used as heterologous hybridization probes to show the accumulation of HRGP transcripts in elicitor-treated bean cells (39). We report here the identification of three classes of bean HRGP cDNA clones containing sequences complementary to elicitor-induced transcripts. These sequences encode distinctive tyrosine-rich proteins containing characteristic Ser-Pro_4 sequences organized in higher-order, tandemly repeated 16-amino-acid units. These specific HRGP transcripts exhibit markedly different patterns of accumulation, one relative to another, in wounded and infected bean hypocotyls. The data indicate the operation of different stress signal systems in, respectively, the early stages of an incompatible interaction, the later stages of a compatible interaction, and in response to wounding, leading to the synthesis of variant HRGPs in these different stress conditions.

MATERIALS AND METHODS

Fungal cultures and elicitor preparation. The source, maintenance, and growth of cultures of *C. lindemuthianum* and generation of conidia were as described by Bailey and Deverall (3). The high-molecular-weight fraction released by heat treatment of isolated mycelial cell walls (1) was used for elicitation of suspension-cultured bean cells.

Plant material. Bean cells were grown in liquid suspension as described previously (16), except that the cultures were maintained in total darkness. Experiments were conducted with 7- to 10-day-old cultures, when the conductivity of the medium was between 2.5 and 2.8 mhos. Germination and growth of French bean (*P. vulgaris* cv. Kievitsboon Koekoek), inoculation of hypocotyls from 8-day-old seedlings with spores of *C. lindemuthianum*, and dissection of site 1 and site 2 tissue samples were described previously (5, 6). Hypocotyls used in wounding experiments were obtained from 10-day-old etiolated seedlings. Tissue was wounded by razor excision to give 5-mm sections which were incubated in 5 mM sodium phosphate (pH 5.5) in darkness.

RNA and DNA isolation. Total cellular RNA was isolated and purified as previously described (6). Poly(A)$^+$ RNA fractions were purified by oligo(dT)-cellulose chromatography. Genomic DNA was isolated from leaves and stems of light grown plants by the cetyltrimethylammonium bromide extraction method of Murray and Thompson (33).

cDNA library construction and screening. Double-stranded cDNA was generated from poly(A)$^+$ RNA by a modification (21) of the method of Okayama and Berg (34). cDNA was cloned into pUC19 (42) at the *Pst*I site by the G-C tailing-annealing procedure (30). Recombinants were recovered by transformation into *Escherichia coli* TB1 by the method of Hanahan (23). Approximately 20,000 recombinant molecules were generated. Colonies were screened by high-density colony hybridization (24) with nick-translated tomato genomic HRGP sequences.

Nucleic acid hybridizations. Hybridization probes were prepared by labeling cDNA sequences by nick translation (35). Hybridization of probes to Southern, RNA, and colony blots was performed in 50% formamide, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, 100 μg of single-stranded salmon sperm DNA per ml, and 1 to 5× Denhardt solution at 42°C. Final washes for hybridization of the tomato HRGP probe to the cDNA library were performed at 50°C in 2× SSC–0.1% sodium dodecyl sulfate. Final washes after hybridization of the bean cDNA clones to Southern and RNA blots were routinely performed at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate.

Nucleic acid sequencing. cDNA sequences were determined by the dideoxy chain-termination method (37) and the chemical degradation method (31). Nuclease *Bal* 31 deletions of clones followed by subcloning with pUC and M13 vectors provided complete sequences of the cDNA clones.

RESULTS

cDNA clones of elicitor-induced HRGP transcripts. A 10-kilobase (kb) HRGP genomic clone (Tom5) from tomato cells had previously been used to show that at least three putative HRGP transcripts accumulate in bean cell cultures after treatment with fungal elicitor (39). To investigate the structure and regulation of these transcripts we generated a plasmid-based cDNA library from poly(A)$^+$ RNA isolated from cells at the time of maximum accumulation, 38 h after the addition of elicitor (39). Approximately 50 putative bean HRGP cDNA clones were identified by colony hybridization with a 3.6-kb *Bam*HI-*Hin*dIII fragment from the Tom5 clone as a probe. Restriction enzyme and Southern blot analysis verified that these clones contained HRGP-homologous inserts (data not shown).

These cDNA clones were grouped into three classes based upon their hybridization to different-sized elicitor-induced transcripts (Fig. 1). Clones Hyp3.6, Hyp2.13, and Hyp4.1 represent the largest cDNA clones from each of the three classes, with inserts of 1.1, 1.6, and 0.7 kb, respectively, and

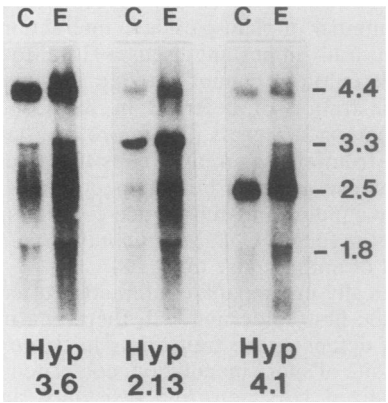

FIG. 1. Accumulation of specific HRGP transcripts in elicitor-treated bean cells. Total cellular RNA was isolated from cells 24 h after elicitor treatment (E) or from equivalent mock-treated control cells (C). Northern blots (5 μg of RNA per lane) were hybridized to ^{32}P-labeled bean HRGP cDNA sequences (Hyp3.6, Hyp2.13, Hyp4.1). Transcript sizes were determined by comparison with the migration of coelectrophoresed RNA size standards (Bethesda Research Laboratories, Gaithersburg, Md.).

were chosen as representatives for further characterization. These three clones hybridized predominantly to transcripts of 4.4, 3.3, and 2.5 kb, respectively (Fig. 1). Each clone also weakly cross-hybridized to the other two transcripts of this set as well as to an additional RNA of 1.8 kb. These four transcripts were present at relatively low abundance in untreated cell cultures and markedly increased in abundance in response to the fungal elicitor (Fig. 1).

A previous study that had used heterologous tomato HRGP sequences to identify three elicitor-induced HRGP mRNAs in beans had determined that the mRNA sizes were 5.6, 2.7, and 1.6 kb based upon their electrophoretic migration relative to rRNA size markers (39). In the present study we used a more complete set of RNA size standards with which to establish the sizes of these transcripts (Fig. 1). The 3.3-kb Hyp2.13 mRNA corresponds to the previously designated 2.7-kb mRNA, since the uniquely characteristic pattern of accumulation of these mRNAs upon fungal infection of bean hypocotyls are similar (39) (see Fig. 6).

Nucleotide sequence of HRGP cDNA clones. The nucleotide sequences of Hyp2.13, Hyp3.6, and Hyp4.1 were determined (Fig. 2). Each sequence contains an open reading frame that encodes numerous Ser-Pro_4 units characteristic of the apoprotein of HRGPs. However, in these three clones, the Ser-Pro_4 units are present within tyrosine-rich, higher-order repetitive units of 16 amino acids that are tandemly reiterated (Fig. 2 and 3).

Hyp2.13 and Hyp3.6 represent the 3′ portions of two different HRGP genes. Each of these sequences contains a 3′-noncoding region in addition to terminal poly(A) sequences. Hyp2.13 contains 12 copies of a 16-amino-acid repeat that has the consensus sequence Tyr_3-Lys-Ser-Pro_4-Ser-Pro-Ser-Pro_4. This sequence is tandemly reiterated, except for the occurrence of a Ser-Pro-Ser-Pro_3 sequence (amino acid position 247 in Fig. 3) within one of the full-length hexadecapeptide repeats. There are also five partial copies of this repeat just upstream of the full-length copies. The translation termination codon at nucleotide position 1106 is followed by a 398-base-pair (bp) noncoding region that is distinctly different from the preceding proline-rich coding sequences. Immediately upstream of, and in frame with, the proline encoding sequence is a 327-bp open reading frame that encodes a non-proline-rich domain (Fig. 2 and 3).

Hyp3.6 contains eight tandemly reiterated copies of the same 16-amino-acid repeat that is found in Hyp2.13 (amino acid positions 94 through 109 can be considered to comprise one repeat; Fig. 3). Whereas the 5′ sequences encode four exact copies of the Try_3-Lys-Ser-Pro_4-Ser-Pro-Ser-Pro_4 consensus sequence, the 3′ sequences encode four imperfect 16-mer repeat units. These eight full-length repeats are followed by two truncated imperfect repeats. The translation termination codon at position 728 is followed by a 384-bp noncoding region that is distinctly different from the preceding proline-rich coding sequence. One unexpected feature of the DNA sequence of Hyp3.6 is the presence of nine translation termination codons preceding, and in frame with, the putative HRGP-coding sequence (Fig. 2). The sequence of this region was therefore verified by both dideoxy chain-termination and chemical degradation sequencing methods. This cDNA may represent an unspliced form of a Hyp3.6 precursor mRNA, in which case the termination codons lie within a putative intron. Two potential intron splice sites lie between these stop codons and the sequence encoding the domain rich in tyrosine and proline (Fig. 2). A restriction fragment corresponding to the 5′ 184 bp of Hyp3.6, and thus containing most of the putative intron, failed to detectably hybridize to any mRNAs in Northern blot (RNA blot) experiments (data not shown). This result is consistent with the hypothesis that the Hyp3.6 cDNA was derived from a rare, unspliced precursor of the Hyp3.6 mRNA that would not be expected to be detected in Northern blots of total cellular RNA, and that the 5′ sequences represent an intron. Interestingly, restriction enzyme mapping of a genomic clone corresponding to Hyp2.13, the cDNA clone that is highly homologous to Hyp3.6, indicates the presence of an intron within that region of the Hyp2.13 gene that corresponds to the part of the Hyp3.6 clone containing the nine termination codons (N. Sauer, unpublished observations).

The close similarity between Hyp2.13 and Hyp3.6 is reinforced by comparison of the nucleotide sequences, which reveals not only 75% homology between the respective coding regions but also the same degree of homology in the 3′ noncoding regions. These observations suggest that the genes encoding Hyp2.13 and Hyp3.6 are closely related and that the conserved 3′ noncoding regions of these genes may have a specific functional significance.

Hyp4.1, in contrast to Hyp2.13 and Hyp3.6, does not contain terminal poly(A) sequences or a presumptive 3′ noncoding region. The open reading frame contains two potential translation initiation codons at positions 6 and 18 near the 5′ end of the clone. The second of these initiation codons is followed by a 28-amino-acid sequence which is rich in hydrophobic and nonpolar amino acids and hence resembles signal sequences in various eucaryotic secretory proteins (41). After this putative transit peptide there is a 200-amino-acid proline-rich domain which contains 12 highly conserved and tandemly reiterated copies of the repeat Tyr_3-His-Ser-Pro_4-Lys-His-Ser-Pro_4. Thus the proline-rich domain of Hyp4.1 is also arranged in 16-mer repeat units. Moreover, the organization of the Tyr_3 and the two Ser-Pro_4 blocks within the 16-mer repeat unit encoded by Hyp4.1 is identical to that within the 16-mer repeat units encoded by Hyp2.13 and Hyp3.6. The Hyp4.1 consensus repeat differs only at two specific locations in which, respectively, His is substituted for Lys and Lys-His is substituted for Ser-Pro compared with the Hyp2.13 and Hyp3.6 sequences. The full-length repeats in Hyp4.1 are preceded by one incomplete variant. Thus, Hyp4.1 encodes a putative HRGP that contains a 200-amino-acid proline-rich domain at the N terminus (Fig. 3).

HRGP sequences in the bean genome. The patterns of hybridization of the three HRGP cDNA clones to Southern blots of bean genomic DNA digested with three different restriction enzymes are shown in Fig. 4. In each case, hybridization at high stringency yielded only one or two autoradiographic bands, and the patterns of hybridization with the three different cDNA clones were entirely different. These results show that each cDNA clone represents a different gene which is present in a single copy or low number of copies in the haploid genome. Analysis at lower stringency revealed weaker hybridization to several additional fragments of genomic DNA, suggesting the existence of other HRGP-related sequences within the bean genome (data not shown).

Differential accumulation of HRGP transcripts in response to fungal infection. The Hyp2.13, Hyp3.16, and Hyp4.1 sequences were used as hybridization probes to monitor changes in the levels of the corresponding transcripts during race-cultivar-specific interactions between hypocotyls of French bean (*P. vulgaris* cv. Kievitsboon Koekoek) and the fungus *C. lindemuthianum*, the causal agent of anthracnose. After inoculation of hypocotyls with spores of either the

TRANSLATED SEQUENCE OF Hyp3.6

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1    TATCTTTCATTCAAGAAACTGTAAACAACGGGATATATAGGTCAAAACCACGGAACAGCAG   60
     Y  L  S  F  K  K  L  *  T  T  G  Y  I  G  Q  N  H  E  Q  Q

61   GACCACAGAACCACGGGACAAACATCACCCAAAATAGAACACCTAATTTTAATCAACTTAA  120
     D  H  R  T  T  D  K  H  H  P  K  *  N  T  *  F  *  S  T  *

121  TCATAAACCCTATTCTACAACACCAAACCCTAGTTCCAGCTACCATCTCCTCCATCGCCT  180
     S  *  T  L  F  Y  N  T  K  P  *  F  Q  L  P  S  P  P  S  P

181  CCACGAGAGTGGCGGGACCCACCATCACCATAACCACGGTCCCTGCCTCCACCGTAACCA  240
     P  R  E  W  R  D  P  P  S  P  *  P  R  S  L  P  P  P  *  P

241  CCACCACCACCACCATCACCAGTTGTTAAGCCACCATACTACTACAAATCTCCACCTCCA  300
     P  P  P  P  P  S  P  V  V  K  P  P  Y  Y  Y  Y  K  S  P  P  P

301  CCATCACCCTCACCTCCCCCTCCATACTACTATAAATCTCCACCCCCACCCTTCACCATCC  360
     P  S  P  S  P  P  P  P  Y  Y  Y  Y  K  S  P  P  P  P  S  P  S

361  CCTCCTCCTCCATACTATTATAAATCACGACCACCACCATCCCCATCACGGCCACCTCCA  420
     P  P  P  P  Y  Y  Y  Y  K  S  P  P  P  P  S  P  S  P  P  P  P

421  TACTATTACAAATCCCCTCCACCTCCATCCCCATCTCCACGCGCCTCCTTATTACTATCAC  480
     Y  Y  Y  K  S  P  P  P  P  S  P  S  P  P  P  P  Y  Y  Y  H

481  AGTCCTCCTCCACCATCTCCCACTCCTCATCCTCCCTACTACTACAAATCCCCTCCACCA  540
     S  P  P  P  P  S  P  T  P  H  P  P  Y  Y  Y  Y  K  S  P  P  P

541  CCAACGTCCTATCCTCCACCTCCTTACCACTATGTGAGCCCTCCACCACCTTCTCCATCT  600
     P  T  S  Y  P  P  P  P  Y  H  Y  V  S  P  P  P  P  P  S  P  S

601  CCACCTCCACCATACTATTACAAATCACCACCTCCTCCCTCCCCAGCCCCCGCTCCAAAG  660
     P  P  P  P  Y  Y  Y  K  S  P  P  P  P  S  P  A  P  A  P  K

661  TACATTTACAAATCACCTCCACCTCCAGCTTACATCTATTCTTCCCCACCTCCACCCATC  720
     Y  I  Y  K  S  P  P  P  P  A  Y  I  Y  S  S  P  P  P  P  I

721  TACAAGTAAGCTCGGATTGTGGAAGCACCATTCACCACAAATCATGTTCTAGTTTTCATG  780
     Y  K

781  GAGGAATAAAGGCAAGCTCCATTTGGAGTGAAGTGAAATATCTATCTTCCTGTCCAAGTT  840

841  CTTTGAATAATAAAAGTCTGAATGTGTCATCTCTGGGCTACAATGTTTGTGATTCAGACC  900

901  CGGTTGGTTCAATTTGTTTGATGGCCATCTTGCACGTCCTGATCTTATTGTGTTCAAGTG  960

961  ACGGACAATGAAAAAGAGGAAGAGAGATTTAAAGTGTAAATGCCAAGTGAAAGTGAAAAA 1020

1021 GTACAGTCCACTCTTAGAGATCTCTTAAATTTATTTGTTTATAGGCCGTCTATGATTGCT 1080

1081 TTTCTATTTATTGTGGAATTTTTGTATTGTCCAAAAAAAAAAAAAAAAAAAAAAAAA 1137
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TRANSLATED SEQUENCE OF Hyp4.1

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1    CAAACATGGGTTTTCCAATGGCCTCTCTCATTCTATCTACTCTTGCATTAACACTAATTT   60
     N  M  G  F  P  M  A  S  L  I  L  S  T  L  A  L  T  L  I  S
                ▲  ▲  ▲  ▲     ▲  ▲  ▲     ▲  ▲  ▲     ▲  ▲

61   CTCTCTTCCCATCCCAAACATTGGCTGACAACTACATCTATTCATCTCCACCACCACCAC  120
     L  F  P  S  Q  T  L  A  D  N  Y  I  Y  S  S  P  P  P  P  P
     ▲  ▲  ▲        ▲  ▲

121  CAAAGCCTTACTACTACCAATCTCGTCCACGACCAAAACACTCACCACGCTCGTCCATACT  180
     K  P  Y  Y  Y  Q  S  P  P  P  P  K  H  S  P  P  P  P  Y  Y

181  ATTACCACTCTCCACGCTCCACCAAAACACTCACCGCCGTCCTCGTTATTACTACCACTCTC  240
     Y  H  S  P  P  P  P  K  H  S  P  P  P  P  Y  Y  Y  H  S  P

241  CCCCACCACCAAAGCATTCACCTCCTCCTCCTTATTACTACCACTCTCCACCTCCACCTA  300
     P  P  P  K  H  S  P  P  P  P  Y  Y  Y  H  S  P  P  P  P  K

301  AGCACTCACCGTCCTCCTCCTTATTACTACCACTCTCCACCACCACCAAAGCATTCACCTC  360
     H  S  P  P  P  P  Y  Y  Y  H  S  P  P  P  P  K  H  S  P  P

361  CTCCACCCTATTACTACCACTCTCCACGTCCACCTAAACACTCACGTCCTCCACCCTATT  420
     P  P  Y  Y  Y  H  S  P  P  P  P  K  H  S  P  P  P  P  Y  Y

421  ACTACCACTCTCCACCACCACCAAAGCATTCACCTCCTCCACCCTATTACTACCACTCTC  480
     Y  H  S  P  P  P  P  K  H  S  P  P  P  P  Y  Y  Y  H  S  P

481  CACCTCCACCTAAACACTCACCTCCTCCACCCTATTACTACCACTCTCCACCACCACCAA  540
     P  P  P  K  H  S  P  P  P  P  Y  Y  Y  H  S  P  P  P  P  K

541  AGCATTCACCTCCTCCCCCATATTACTACCACTCTCCACCCCCACCAAAGCATTCACCTC  600
     H  S  P  P  P  P  Y  Y  Y  H  S  P  P  P  P  K  H  S  P  P

601  CTCCTCCTTATTACTACCACTCTCCCCCACCACCTAAGCATTCACCTCCTCCACCCTATT  660
     P  P  Y  Y  Y  H  S  P  P  P  P  K  H  S  P  P  P  P  Y  Y

661  ACTACCACTCTCCACCTCCACCTAAACACTCA  692
     Y  H  S  P  P  P  P  K  H  S
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TRANSLATED SEQUENCE OF Hyp2.13

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1    AGGTGCCACTGTCGAAGTGACTTGTGAAGTTGGAGGCAAGACCATAAAGTCTTACGGTAA   60
     G  A  T  V  E  V  T  C  E  V  G  G  K  T  I  K  S  Y  G  N

61   CACTAAGAGCAATGGGAAATACAGCATCACTGTCGAAGGATTGGACTATGTTAAATATGG  120
     T  K  S  N  G  K  Y  S  I  T  V  E  G  L  D  Y  V  K  Y  G

121  AGGCACAGTCTGCAAGGCTCAACTCCATGCTCCTCCTAAGGGCTCACGTTGCAGCATACC  180
     G  T  V  C  K  A  Q  L  H  A  P  P  K  G  S  R  C  S  I  P

181  CACCAAGCTTAACGAGGGAACCAAGTTGGCACTGAAGTCCAAGGATAAGTACGAGGTTGT  240
     T  K  L  N  E  G  T  K  L  A  L  K  S  K  D  K  Y  E  V  V

241  TCTGAAGGCTAAGCCTTTTGCTTATGCTCCTAAAAAGCCTTATGATTGTGAAAAGCACAA  300
     L  K  A  K  P  F  A  Y  A  P  K  K  P  Y  D  C  E  K  H  K

301  GCATTCACCCACTCCTTATCACAAACCATACTACTACAACTCTCCCCCACCACCATACTA  360
     H  S  P  T  P  Y  H  K  P  Y  Y  Y  N  S  P  P  P  P  Y  Y

361  CTATAAGTCCCCTCCTTACTACTACAAGAGCCCCCCCTCCACCATCACCATCTCCTTCTCC  420
     Y  K  S  P  P  Y  Y  Y  K  S  P  P  P  P  S  P  S  P  S  P

421  ATACTACTATAAATCTCCACCTCCTCCACACAAGGATCCTTATTATCCTCCTTACTACTA  480
     Y  Y  Y  K  S  P  P  P  P  H  K  D  P  Y  Y  P  P  Y  Y  Y

481  CAAATCACGTCCTCCACCCTCCCCATCACGTCCACCACCCTACTACTACAAGTCTCCTCC  540
     K  S  P  P  P  P  S  P  S  P  P  P  P  Y  Y  Y  K  S  P  P

541  ACCACGCATCTCCATCACCACCACCACGTTACTACTACAAATCACGTCCACCACCAGATCC  600
     P  P  S  P  S  P  P  P  P  Y  Y  Y  K  S  P  P  P  P  D  P

601  ATCACCACCACGTCCCTACTACTACAAGTCACGTCGTCCTCCATCACGTTCTCCACCACC  660
     S  P  P  P  P  Y  Y  Y  K  S  P  P  P  P  S  P  S  P  P  P

661  ACCATACTACTACAAATCACGTCCACCACCATCTCCCTCACCACCACGTCCCTACTACTA  720
     P  Y  Y  Y  K  S  P  P  P  P  S  P  S  P  P  P  P  Y  Y  Y

721  CAAGTCCCCACCACCACCTTCCCCTTCACCACCACCATCCCCATCACCACCACCTCCCTA  780
     K  S  P  P  P  P  S  P  S  P  P  P  S  P  S  P  P  P  P  Y

781  CTACTACAAATCACGTCCACCACCGGATCGTTCACCGCCACCTCGTTACTATTACAAGTC  840
     Y  Y  K  S  P  P  P  P  D  P  S  P  P  P  P  P  Y  Y  Y  K  S

841  ACCACCACCACCATCTCCATCACCACCACCACCCTACTACTACAAGTCACCTCCTCCTCC  900
     P  P  P  P  S  P  S  P  P  P  P  Y  Y  Y  K  S  P  P  P  P

901  ATCACCTTCTCCACCACCTCGTTACTACTACAAGAGCCCTCCTCCACCATCTCCCGATCC  960
     S  P  S  P  P  P  P  Y  Y  Y  K  S  P  P  P  P  S  P  D  P

961  TCCTACTCCCTACTACTACAAATCTCGTCCACCACCATCACCGTCTCCCCCACCTCCTTA 1020
     P  T  P  Y  Y  Y  K  S  P  P  P  P  S  P  S  P  P  P  P  Y

1021 TTACTACGTGAGTCCCCCACCACCAACCAAGTCTCCTCCACCACCGGCATACAGCTATGC 1080
     Y  Y  V  S  P  P  P  P  T  K  S  P  P  P  P  A  Y  S  Y  A

1081 TTCCCCTCCACCACCCACCTACAACTAAGCTTCGAGTTAAGCAGAAACGACGATAAGTCA 1140
     S  P  P  P  P  T  Y  N

1141 TGTTCTAGTATTCATTGTGGAATAAAATCAGGTTCCATTTGGAGCAGGGTTAAGCATCTA 1200

1201 ATTTGTTGTAAAAGTTCTTTGAATAATAAGAGTCTGAATGTATCGTCTCTGAGCTACATC 1260

1261 TTCACGATTCGGATGAGGCTGCTTCTATTTATTTGGTGGTGATCTGCACATCTGATCTAA 1320

1321 TAAGTTCGAGGGAGCGACAATGAAAACGAGGAACCAAGATTTCAAATCCTGATGTGGAGG 1380

1381 CTGCTCTTAGAGATCTCTTCATTTCTTTCTTTGTAGATTACATATATATTTGATCGTGTA 1440

1441 ATGATTGCATTCAAAGTATTGTCAAATTCTTTTCATTATATATCATCTTCTTCTGTTCTT 1500

1501 TGCAAAAAAA 1510
```

FIG. 2. Nucleotide sequences and derived amino acid sequences of partial-length HRGP cDNA clones. The boxed amino acid sequences are the consensus sequences of the hexadecapeptide repeats found within each clone. The heavy arrows in Hyp3.6 and Hyp2.13 mark the boundaries between tyrosine- and proline-rich and -poor domains. Stop codons of the HRGP-coding sequences are underlined, and stop codons within the presumptive intron in clone Hyp3.6 are represented by asterisks (*). Two potential 3′ intron-exon junctions (AG) in Hyp3.6 are overlined. Arrowheads in Hyp4.1 mark hydrophobic and nonpolar amino acids that lie within a putative transit signal sequence. Circles denote methionine residues representing potential translation start sites.

Hyp 3.6

```
  1  PPPPPPSPVVKPP
 14  YYYKSPPPPSPSPPPP
 30  YYYKSPPPPSPSPPPP
 46  YYYKSPPPPSPSPPPP
 62  YYYKSPPPPSPSPPPP
 78  YYYHSPPPPSPTPHPP
 94  YYYKSPPPP
103  TSYPPPP
110  YHYVSPPPPSPSPPPP
126  YYYKSPPPPSPAPAPK
142  YIYKSPPPPA
152  YIYSSPPPPIYK*
```

Hyp 4.1

```
  1  NMGFPMASLILSTLALTLISLFPSQ
 26  TLADN
 31  YIYSSPPPPPKP
 43  YYYQSPPPPKHSPPPP
 59  YYYHSPPPPKHSPPPP
 75  YYYHSPPPPKHSPPPP
 91  YYYHSPPPPKHSPPPP
107  YYYHSPPPPKHSPPPP
123  YYYHSPPPPKHSPPPP
139  YYYHSPPPPKHSPPPP
155  YYYHSPPPPKHSPPPP
171  YYYHSPPPPKHSPPPP
187  YYYHSPPPPKHSPPPP
203  YYYHSPPPPKHSPPPP
219  YYYHSPPPPKHS
```

Hyp 2.13

```
  1  GATVEVTCEVGGKTIKSYGNTKSNG
 26  KYSITVEGLDYVKYGGTVCKAQLHA
 51  PPKGSRCSIPTKLNEGTKLALKSKD
 76  KYEVVLKAKPFAYAPKKPYDCEKHK
101  HSPTPYHKP
110  YYYNSPPPP
119  YYYKSPP
126  YYYKSPPPPSPSPSP
141  YYYKSPPPPHKDP
154  YYPP
158  YYYKSPPPPSPSPPPP
174  YYYKSPPPPSPSPPPP
190  YYYKSPPPPDPSPPPP
206  YYYKSPPPPSPSPPPP
222  YYYKSPPPPSPSPPPP
238  YYYKSPPPPSPSPPP
253  SPSPPPP
260  YYYKSPPPPDPSPPPP
276  YYYKSPPPPSPSPPPP
292  YYYKSPPPPSPSPPPP
308  YYYKSPPPPSPDPPTP
324  YYYKSPPPPSPSPPPP
340  YYYVSPPPPTKSPPPP
356  AYSYAPPPPTYN*
```

FIG. 3. Amino acid sequences of the HRGP coding regions of the cDNA clones. Repetitive units are arranged in order to display their periodicity. Position of stop codons are marked by asterisks (*).

incompatible race β or the compatible race γ, there was a period of 30 to 40 h before the hyphae first came in contact with the host epidermal cells, during which the spores germinated and the fungus developed an appressorium and infection peg. At this stage in the incompatible interaction (host resistant), the presence of the fungus was rapidly

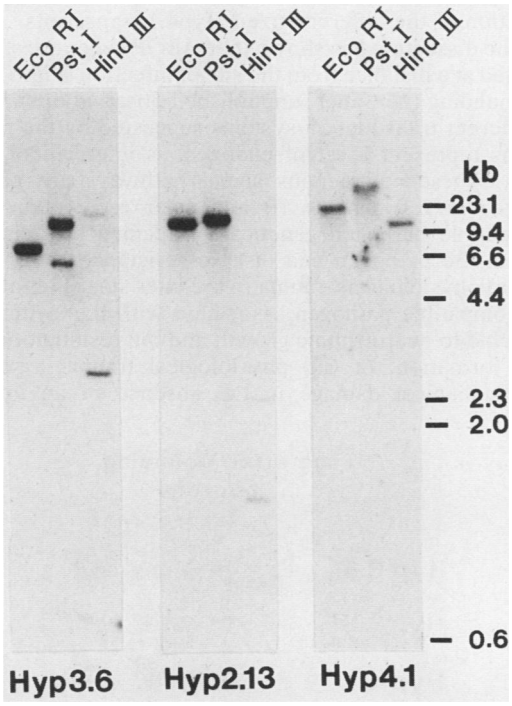

FIG. 4. Southern blot hybridization analysis of HRGP sequences in the bean genome. Genomic DNA from leaves of bean cultivar Tendergreen was digested with *Eco*RI, *Pst*I, and *Hin*dIII. The fragments were separated by electrophoresis in 1% agarose gels, blotted onto nitrocellulose, and hybridized to ^{32}P-labeled bean HRGP cDNA sequences.

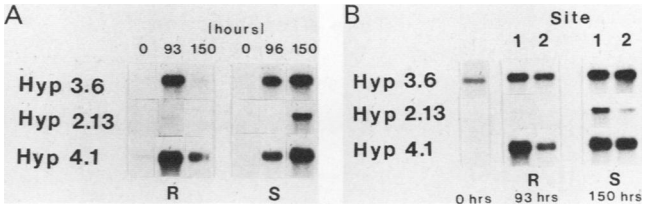

FIG. 5. Accumulation of HRGP transcripts in bean hypocotyls infected with *C. lindemuthianum*. (A) Differential accumulation in an incompatible interaction with race β (host resistant [R]) and a compatible interaction with race γ (host susceptible [S]). RNA was isolated from directly infected tissue (site 1) at approximately 93 and 150 h after spore inoculation. (B) Systemic accumulation of HRGP transcripts. RNA was isolated from directly infected tissue (site 1) and laterally adjacent, uninfected tissue (site 2). Northern blots were hybridized to ^{32}P-labeled Hyp2.13, Hyp3.6, and Hyp4.1 cDNA sequences.

detected, leading to activation of defense responses, localized cell death, and expression of hypersensitive resistance in which further spread of the fungus was completely inhibited. In contrast, in the compatible interaction (host susceptible), the infected cells remained alive and the fungus underwent substantial biotrophic growth, ramifying throughout the tissue. Subsequently, whereas the hyphal tips remained biotrophic, the older regions became necrotrophic, leading to extensive host cell death and development of spreading anthracnose lesions.

Previous studies with the heterologous tomato sequence as a hybridization probe have shown that in the incompatible interaction there is a marked, early accumulation of HRGP transcripts, with maximum levels occurring between 70 and 100 h after spore inoculation. Accumulation of HRGP transcripts occurred not only in directly infected tissue immediately below the site of spore inoculation associated with expression of hypersensitive resistance but also in uninfected tissue at a distance from the site of inoculation. Expression of HRGP genes in distal tissues that became resistant to further infection by normally virulent pathogens suggests that HRGP accumulation may be associated with the establishment of induced immunity. In contrast, in the early stages of the compatible interaction there was only a relatively weak induction of HRGP transcripts, and marked accumulation was not observed until the later stages of the interaction, 150 to 190 h after spore inoculation, correlated with the attempted limitation of developing anthracnose lesions.

In the present study we used Hyp2.13, Hyp3.6, and Hyp4.1 cDNA sequences as hybridization probes under conditions of high stringency to monitor accumulation of these specific transcripts in response to infection. This analysis revealed marked differences between these transcripts in the patterns of accumulation. Thus although the Hyp2.13 transcript accumulated to high levels in tissue immediately under the site of spore inoculation (site 1) in the later stages of the compatible interaction (Fig. 5A, panel S), this transcript was much more weakly induced during any stage of the incompatible interaction (Fig. 5A, panel R; Fig. 6). In marked contrast, both Hyp3.6 and Hyp4.1 transcripts strongly accumulated in tissue under the site of spore inoculation (site 1) in the early stages of the incompatible interaction, to levels comparable to (Hyp3.6) or in excess of (Hyp4.1) the maximum levels attained in the later stages of the compatible interaction (Fig. 6). Moreover, these specific transcripts exhibited different kinetics of accumulation in

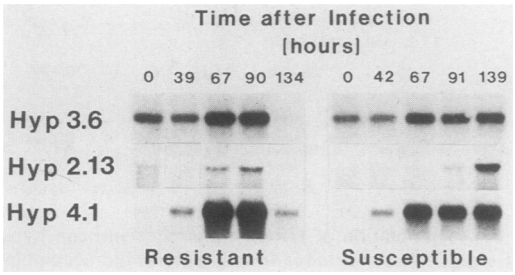

FIG. 6. Kinetics of the accumulation of HRGP transcripts in bean hypocotyls infected with *C. lindemuthianum*. RNA was isolated from directly infected tissue (site 1) at various times after inoculation with spores of the incompatible race β (resistant) or the compatible race γ (susceptible). Northern blots were hybridized with ^{32}P-labeled Hyp2.13, Hyp3.6, or Hyp4.1 cDNA sequences under conditions that gave hybridization only to the respective homologous transcripts.

infected tissue. Thus, in both compatible and incompatible interactions, Hyp4.1 transcript accumulation was first detected as early as 40 h after spore inoculation, whereas Hyp3.6 transcript accumulation was not observed until 67 h in the incompatible interaction and somewhat later in the compatible interaction (Fig. 6). The Hyp2.13 transcript accumulated only after 90 h in the compatible interaction but appeared as early as 67 h in the incompatible interaction (Fig. 6A). All three transcripts accumulated not only in the tissue immediately underlying the site of spore inoculation (site 1) but also in hitherto uninfected tissue at a distance (site 2) (Fig. 5B).

Accumulation of HRGP transcripts in wounded hypocotyls. Differential patterns of accumulation of HRGP transcripts were also observed in excision-wounded hypocotyl tissue (Fig. 7). The wound-induced transcripts homologous to Hyp3.6 and Hyp2.13 were the same size as the corresponding transcripts in elicitor-treated cells and infected hypocotyls. However, a Hyp4.1 mRNA approximately 300 bp smaller than the corresponding infection- or elicitor-induced mRNA was strongly induced upon wounding. In addition, there was relatively weak induction of a Hyp4.1 transcript of the same size as that detected in elicitor-treated cells and infected hypocotyls. Induction of transcripts homologous to the three HRGP cDNA clones was first detected within 1.5 h after wounding. However, whereas Hyp3.6 mRNA was almost maximally induced by this time, Hyp2.13 and Hyp4.1 mRNAs did not reach maximum levels until 12 h after wounding. Hyp3.6 mRNA levels decayed between 12 and 24 h, whereas the other mRNAs remained at maximum levels.

DISCUSSION

We have characterized three different stress-induced transcripts which encode apoproteins of cell wall HRGPs. Two striking features are the highly repetitive structural motifs in the deduced amino acid sequences of the encoded polypeptides and the markedly different patterns of accumulation of these transcripts in wounded and infected hypocotyls.

The stress-induced transcripts are encoded by separate genes, each of which is present at a low copy number within the bean genome. These HRGP genes are differentially regulated in terms of both the kinetics of transcript accumulation and the overall pattern of activation in wounded hypocotyls and hypocotyls infected with an incompatible or a compatible race of the fungus *C. lindemuthianum*. Individual members of several other multigene families are selectively expressed at different stages of development in specific tissues or organs (12, 20, 25). Our data indicate that within the same organ and at the same developmental stage, individual genes within a gene family are selectively activated by different forms of stress. Particularly striking is the marked preferential induction of Hyp2.13 in the compatible interaction compared with the incompatible interaction, whereas Hyp3.6 and Hyp4.1 are strongly induced in both types of interaction. This clear differential pattern implies that there are different cues and signal transduction systems for defense gene activation associated with the expression of localized hypersensitive resistance and induced immunity in the incompatible interaction compared with attempted lesion limitation in the later stages of the compatible interaction.

The different kinetics of accumulation of the HRGP transcripts in wounded tissue likewise implies the operation of complex signal systems in response to mechanical damage. Moreover the accumulation of a smaller Hyp4.1 transcript in wounded tissue compared with that in infected tissue implies that the cues for accumulation of defense gene transcripts in response to wounding are in turn different from the signal systems operating in infected tissue. Accumulation of different-sized Hyp4.1 transcripts in wounded and infected tissue is reminiscent of recent observations in carrots, where different-sized HRGP transcripts accumulate during development and in response to wounding (10). In that case it was shown that the two transcripts arose from the use of alternative start sites for transcription within the same gene. Since there appears to be only one Hyp4.1 gene within the bean genome, by analogy we propose that wounding and infection stimulate initiation of transcription from different sites regulated by different promoters, although we cannot yet exclude mechanisms such as alternative splicing for the generation of the different-sized Hyp4.1 transcripts.

Tissue dissection has shown that HRGP genes are strongly activated at a distance from the site of infection (Fig. 5B) (39) and wounding (N. Sauer, unpublished observations). Hence the different transduction systems suggested by the present analysis represent a set of endogenous intercellular stress signals. These endogenous signal pathways are possibly activated in response to (i) molecular recognition of an incompatible pathogen genetically specified by avirulence genes in the pathogen and disease resistance genes in the host, (ii) physiological trauma in the later stages of infection by a compatible pathogen associated with the switch from biotrophic to necrotrophic growth and the resultant onset of lesion formation, or (iii) physiological trauma associated with mechanical damage in the absence of an invading

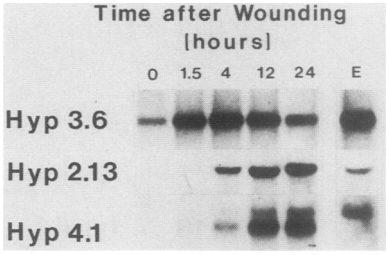

FIG. 7. Differential accumulation of HRGP transcripts in excision-wounded hypocotyl tissue. RNA was isolated from bean hypocotyls at increasing times after wounding. Northern blots were hybridized to ^{32}P-labeled bean HRGP cDNA sequences. Lane E shows the relative migration of elicitor-induced transcripts as standards.

microorganism. The operation of several distinct signal systems would allow the plant to activate similar arrays of defense responses in very different biological circumstances, related to the expression of different forms of disease resistance including localized hypersensitivity, induced immunity, and lesion limitation as well as prevention of infection after mechanical damage.

Isolation and characterization of the *cis*-acting sequences and *trans*-acting regulatory proteins responsive to the different cues delineated in the present study will provide a direct approach for analysis of the molecular mechanisms underlying these intercellular signal transduction systems. Based on activity in bioassays for induction of various defense responses, a number of candidates for intercellular stress signal molecules have been proposed, including the gaseous hormone ethylene, endogenous plant cell wall oligosaccharide elicitors, and glutathione (7, 14; V. Wingate and C. Lamb, unpublished data). However in no case has definitive evidence been obtained for a causal role in intercellular signaling of stress. Expression in transgenic cells of chimeric genes encoding reporter proteins such as chloroamphenicol acetyltransferase regulated by the appropriate stress-responsive HRGP promoters will provide a bioassay for molecules involved in the signal pathways that, unlike conventional bioassays, will be rapid, sensitive, and specific to particular signal pathways and hence will allow purification and characterization of the messengers.

Ser-Pro_4 is the characteristic structural feature of the primary translation product of cell wall HRGP apoproteins, the proline residues of which are subsequently hydroxylated to give the Ser-Hyp_4 units found in the mature glycoproteins. The stress-induced transcripts characterized here encode HRGPs in which these Ser-Pro_4 units are organized within remarkable 16-mer repeat units. Partial amino acid sequence analysis of fragments derived from soluble HRGP molecules in tomatoes has shown that Ser-Hyp_4 units are found in a number of characteristic peptide sequences that are consistent with the existence of higher-order repeat units within these proteins (40). However difficulties associated with amino acid sequencing of such repetitive, hydroxyproline-rich polypeptides has precluded analysis of the abundance and organization of putative repeat units. The amino acid sequences deduced from recent nucleotide sequence analysis of tomato and carrot HRGP cDNA and genomic clones has confirmed that Ser-Pro_4 units are to some extent organized within higher-order repeats, distributed throughout the proline-rich domains of the apoprotein (10, 39). However, the polypeptides encoded by the stress-induced HRGP transcripts described herein exhibit a number of novel and noteworthy features, including the arrangement of two Ser-Pro_4 units at specific locations within a 16-amino-acid canonical unit, the precise conservation of amino acid sequence in these repeat units, and the contiguous arrangement of the repeat units in tandem with few intervening amino acid residues.

A particularly striking feature of the polypeptides encoded by these stress-induced HRGP transcripts is the high level of tyrosine organized almost exclusively in Tyr_3 blocks located at a precise site in the canonical repeat unit. Although tyrosine is a characteristic component of all known cell wall HRGPs, this amino acid usually occurs as single residues distributed throughout the polypeptide or in the configuration Tyr-Lys-Tyr-Lys (40). Before this report only one case of an HRGP containing Tyr_3 blocks had been reported (18, 26). However, the abundance and organization of the Tyr_3 units could not be determined from analysis of the available peptide fragments. Tyrosine is involved in the formation of intramolecular isodityrosine cross-links in HRGPs, and it has been proposed that tyrosine is also involved in oxidative intermolecular cross-linkages (18, 19). Hence we propose that the high levels of tyrosine, organized in Tyr_3 blocks that are regularly distributed throughout the polypeptides encoded by stress-induced bean HRGP transcripts, may be closely related to the functions of these particular HRGPs in plant defense. Specifically, the abundant Tyr_3 blocks may facilitate erection of a structural barrier to infection by extensive intra- and intermolecular HRGP oxidative cross-linking and provision of sites for anchorage and elaboration of stress-induced lignin as a further structural barrier to infection. Moreover, these Tyr_3 blocks may promote direct inactivation of invading microorganisms mediated by oxidative tanning reactions of microbial surface proteins (38).

Whereas the 16-mer repeat units exhibit identical dispositions of the Tyr_3 and Ser-Pro_4 blocks, there are marked differences at the remaining sites such that the Hyp4.1 repeat contains His and Lys-His, respectively, compared with Lys and Ser-Pro in Hyp2.13 and Hyp3.6. These substitutions will have a considerable effect on the charge properties of the proteins at physiological pH and hence may have functional significance in relation to interactions with other charged polymers in the cell wall as well as the surfaces of invading microorganisms. These variant forms, encoded by genes responsive to different cues, may be optimally attuned for defense functions under different stress conditions.

We have recently identified a fourth HRGP cDNA clone complementary to a transcript that is not induced by elicitor or infection. Interestingly, the polypeptide encoded by this transcript is not organized around the 16-mer canonical repeat and does not contain Tyr_3 blocks (N. Sauer and C. J. Lamb, manuscript in preparation), suggesting that these features of the proteins encoded by the three transcripts described here are specifically related to their functions in plant defense.

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